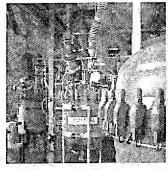
enhancing medicine's future

SHEARWATER®



Shearwater Corporation

CATALOG 2001

Polyethylene Glycol and Derivatives
for Biomedical Applications

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enhancing medicine's future

SHEARWATER®

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Cover image
Pilot plant for production of PEG reagents in batch sizes up to 20 kg.

Introduction to Shearwater Corporation

Shearwater Corporation's PEGylation technology offers the healthcare industry solutions to common drug delivery problems such as poor solubility, stability, short pharmacokinetic half-life and unwanted immunogenicity. The Company's customers, which include Amgen, Roche, Pharmacia, Calltech, DuPont, Regeneron, Serono, Maxygen and United Therapeutics, have demonstrated through numerous human clinical trials that the use of Shearwater's proprietary reagents provide a safe and effective solution to these common problems.

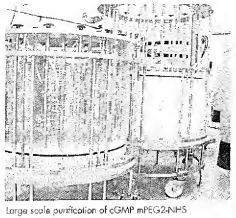
Shearwater provides two options to its customers:

Option 1 - Choose one of the proven activated poly(ethylene glycol) (PEG) derivatives featured in this catalog for coupling to your therapeutic, diagnostic or medical device candidate;

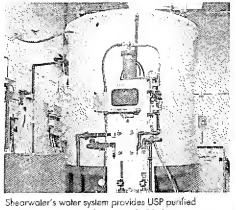
or

Option 2 - Let Shearwater's team of world-class experts optimize or create the solution for you.

Through these options, Shearwater offers its customers a one-stop-shop for PEGylation, including basic research leading to the creation of proprietary, optimized PEG reagents, full-scale cGMP manufacturing and a full regulatory package including the Drug Master File. Currently the Company's corporate partners have ten PEG-products utilizing Shearwater's proprietary technology in 14 clinical trials. Three of these products have completed trials and are in the approval stage, and one has recently received regulatory approval in the U.S.



Large scale purification of cGMP mPEG2-NHS



Shearwater's water system provides USP purified water to support manufacturing of PEG-derivatives.

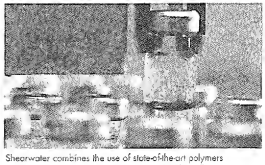
Shearwater's Facilities

Shearwater Corporation currently employs more than 90 personnel located in two facilities in Huntsville, Alabama. The Company's Research group is located in 11,000 sq. ft. at 2305 Spring Branch Road, and is ideally set up to perform research into PEG chemistry and the PEGylation of all forms of drug substance, including proteins, peptides and small molecules. In addition, the group has a number of other PEG-based technologies, such as hydrogels and unimolecular micelles, which aid in the delivery of difficult drugs; e.g. those which are insoluble in aqueous systems, or which have short pharmacokinetic half-lives.

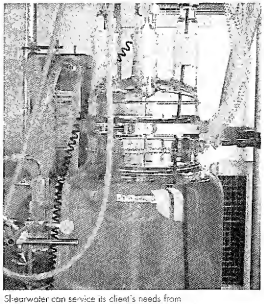
The Company's current headquarters are located in 35,000 sq. ft. at 1112 Church Street, where Administration, Development and Manufacturing (both catalog and cGMP) are housed. A third 50,000 sq. ft. facility is currently under construction at 490 Discovery Drive, and is due for completion in June 2001. This facility, which will become the Company's new headquarters, will house Administration, Research and additional cGMP Manufacturing.

cGMP Manufacturing of Activated PEG Derivatives

Of particular importance to customers who intend to commence human clinical testing of PEG-drugs followed by commercialization, are the Company's cGMP Manufacturing facilities. Currently, Shearwater has three state-of-the-art production suites, which can manufacture any of the Company's reagents in batch sizes of up to 10 kg. In addition, the Company has one pilot production plant that can produce batches of activated PEG derivatives of up to 20 kg. Five new cGMP production areas are being built at the Company's Discovery Drive facility, which will commence cGMP production in the fourth quarter of 2001. Batch size capacity will increase to 80 kg at that time, and the Company's customers will have the peace of mind of knowing that two, physically separate manufacturing facilities will be producing cGMP PEG-derivatives.



Shearwater combines the use of state-of-the-art polymers and organic chemistry technologies to provide well characterized PEG-derivatives.



Shearwater can service its client's needs from fundamental research through full-scale commercial manufacturing by providing PEG-derivatives in 100 g through 20 kg quantities.

cGMP Manufacturing of Active Pharmaceutical Ingredients

At the present time, the Company's cGMP facilities are qualified to produce activated PEG derivatives. Plans are in preparation to commence construction at the Company's Discovery Drive facility of two clean room facilities which will be qualified to produce bulk small molecule and peptide active pharmaceutical ingredients (API). PEGylation of such APIs will also be conducted to cGMP, making Shearwater Corporation the only company to offer a complete service from fundamental research through to cGMP production of bulk final PEG-drug substance. This capability is projected to go online in the first half of 2002.

Second-Generation PEGylation Chemistry

Much protein PEGylation work has been conducted with "first-generation" PEGylation chemistry characterized by: restriction to low MW mPEG because of high diol content generally observed for high MW mPEGs; crosslinking and aggregation from difunctional contaminant; hydrolytically unstable linkages; low selectivity and side reactions. Examples of first-generation mPEG derivatives are succinimidyl succinate (or SS), succinimidyl carbonate (or SC), and tresylate. Coupling of mPEG-SS with lysine groups on a protein produces stable amide linkages, but the backbone ester group hydrolyses readily to produce the protein with an attached immunogenic hapten. Similarly, PEGylation with mPEG-SC has been shown to be accompanied by significant amounts of Lossen rearrangement that leads to loss of reagent and can lead to coupling of beta-alanine to the protein[27]. Also mPEG-SC has been shown to attach to imidazole rings of histidine groups to produce a hydrolytically unstable urethane[28]. PEGylation with tresylate has been shown to be accompanied by nucleophilic attack on fluorine resulting in formation of a degradable sulfonamide[29]. All three derivatives are limited to low MW mPEGs because of the presence of large amounts of diol PEG in high MW mPEGs (resulting from presence of water during polymer formation). Also, low MW mPEGs with first-generation active groups are highly unselective because the relatively small mPEG can penetrate into otherwise poorly accessible regions on the protein surface.

The second-generation chemistry that Shearwater uses has been designed to avoid the aforementioned problems. A typical example is the Company's mPEG2-NHS reagent. This compound is prepared by coupling mPEG to lysine to produce mPEG2-COOH, which can be purified by ion-exchange chromatography to give a pure monofunctional acid. The acid can be activated by formation of the succinimidyl active ester. Because of this purification step, high purity mPEG2-NHS can be prepared with MW to 60,000. The highly crowded reactive group leads to greatly improved selectivity, as only the most accessible groups on the protein surface are available. Reaction with lysines produces a stable amide linkage. Similarly, mPEG2-MAL is selective for thiol groups, and mPEG2-ALD is selective for the N-terminus under optimized attachment conditions. Other examples are described in the following pages.

Benefits of Second-Generation PEGylation

Attachment of Shearwater's activated PEG derivatives to pharmaceutical products typically yields the following benefits:

- Improved solubility and stability
- Reduced immunogenicity and proteolysis
- Reduced clearance from the body
- Renal filtration slowed by increased molecular size
- Carbohydrate and peptide receptor clearance mechanisms "fooled" by PEG's "cloaking" ability
- Less frequent dosing required due to greatly increased circulation time
- Optimized biodistribution in some cases
- More of the administered dose is available to reach the target
- Improved efficacy due to increased concentration and longer dwell time at site of action

Case Histories

Clinical Performance of Shearwater's Second-Generation PEGylation

The PEGylation of interferon-alfa in collaboration with Hoffmann-La Roche illustrates the power of Shearwater's second generation PEGylation technology. Interferon-alfa ("IFN") has been the prime drug for treatment of hepatitis C for several years. Unfortunately, IFN has poor efficacy, with only a small percentage of patients showing sustained viral elimination after cessation of treatment[1,2,3]. The primary problem with the drug is that the blood circulation half-life is only a few hours. Thus, for the standard program of three injections per week, the hepatitis C virus has extended periods of time when it is not exposed to IFN.

Roche first PEGylated its proprietary IFN (Roferon, interferon-alfa-2a) with mPEG 5000 (using an active carbonate to produce a urethane-linked PEG, a first-generation PEGylation technique) and conducted clinical trials on the conjugate[4-7]. However, the clinical trials were not successful and the product was never marketed. The reason for failure of the 5000 Da conjugate was that the blood circulation half-life for the conjugate was only slightly improved relative to that of the native protein.

The next step in the Roche development program was to collaborate with Shearwater to examine various second-generation PEGylation chemistries. Suffice it to say that the "winner" in the investigation was IFN coupled to Shearwater's mPEG2-NHS 40,000. Since the protein has a MW of about 19,000, the mPEG-IFN conjugate is a much larger molecule. Animal studies and modeling indicated that this conjugate (given the trade name PEGASYS®) would have the desired pharmacokinetics and activity. In particular, it was predicted that the steady state blood concentration would be essentially constant. In fact, the modeling data were confirmed by human clinical trials resulting in essentially constant blood concentrations being achieved by once-weekly subcutaneous injections[8]. PEGylation with mPEG2-NHS 40,000 decreases clearance relative to native IFN and gives an elimination half-life of 77 hours instead of 9 hours. In addition, PEGASYS exhibits a low volume of distribution and sustained absorption from the subcutaneous injection site.

A Phase II human clinical trial with 155 patients where PEGASYS was injected subcutaneously once a week, showed that 62% of patients were free of virus after 48 weeks of treatment, and 36% of patients exhibited a sustained viral clearance 24 weeks later[9]. The optimal dose was 180 mcg/wk. In comparison, native IFN injected three times per week (standard therapy) gave only a 3% sustained response. A recently completed Phase III study with 531 patients supported the Phase II study and showed a sustained response rate of 39% for PEGASYS[10]. An important point, which has been misinterpreted by some, is that the native IFN control group in this study gave an unusually high response rate of 19%. However, this control group, unlike those in all other trials, received

a non-standard, high-dose induction regimen of native IFN for the first 12 weeks of treatment, resulting in approximately twice the typical response rate. A second Phase III study with 271 cirrhotic patients showed 43% of the patients receiving PEGASYS had viral clearance at the end of 48 weeks treatment[3].

The dramatically improved clinical efficacy of PEGASYS over the native IFN led Roche to file for market clearance with the FDA on May 23, 2000.

The Value of Collaborating With Shearwater's Research Group

As the leader in PEG reagent development and cGMP manufacturing, a natural evolution of the Company's skills has been the mastery of applications of PEG technology to drug delivery and medical devices. Early in Shearwater's development, PEGylation was performed by many of the Company's partners in the pharmaceutical and biotechnology industries. In 1997, Shearwater formed a dedicated Research Group and began working closely with partners in the pharmaceutical industry to develop and optimize PEG technologies for use with their drugs or devices. This has proven to be a cost effective and highly successful strategy for the Company's partners. Four of Shearwater's partners in this effort are now either in the clinic or will be starting trials shortly, and a number of projects are now in the preclinical pipeline. These projects cover the range of PEG strategies for small drug molecules, peptides, proteins and hydrogels both for medical devices and drug delivery.

A recent example is the Company's partnership with United Therapeutics, which resulted in new intellectual property on a PEG-prostacyclin analog. A second example is the Company's collaboration with Serono on the PEGylation of interferon-beta. Serono's preferred strategy was to use Shearwater's second-generation, site-specific PEGylation technology. Interferon-beta has a single free -SH group which can be PEGylated with a variety of Shearwater's thiol-specific activated PEG derivatives. Unfortunately, the standard chemistries featured in this catalog proved unsuccessful due to the fact that the thiol group is partially sterically hindered. Shearwater's Research Group solved the problem by first PEGylating the molecule with a low-MW, thiol-specific, bifunctional PEG, specially synthesized for the purpose. Once this had been attached, it became possible to attach a high MW mPEG-THIOL to the remaining functional group on the low-MW PEG. This novel two-step process has now been patented.

Utilizing Shearwater's Research Group brings a broad range of clinically-tested solutions to bear on a variety of drug delivery problems:

Shearwater's Protein Technologies

- ★ site-specific PEGylation
- ★ N-terminal PEGylation
- ★ non-terminal amine PEGylation
- ★ controlled release
- ★ heterobifunctional PEG for targeting

Shearwater's Small Molecule Technologies

- ★ functional group specificity
- ★ multifunctional PEG for higher loading
- ★ controlled release
- ★ heterobifunctional PEG for targeting

Shearwater's Hydrogel Technologies

- ★ biocompatible
- ★ in situ formation
- ★ degradable gels with degradable links to drugs for controlled release
- ★ can link to a variety of functional groups in proteins and small molecules

Shearwater has the most complete and versatile range of PEG technologies in the industry coupled with world-class expertise in the application of these technologies.

Let us apply our delivery technologies to your problem molecule or device and help you move it swiftly and economically from the lab to the clinic.

Custom Synthesis

Shearwater's Research Group contains a team of highly skilled scientists who specialize in the synthesis of activated PEG derivatives. If you do not see what you are looking for in the catalog, please call our Sales team, and we will be happy to discuss ways in which we can help you.

Radio-Labeled PEG and PEG Derivatives

Shearwater Corporation and Perkin Elmer (NEN Life Science Products) have a collaborative agreement to provide Shearwater customers with radio-labeled PEG and PEG derivatives. NEN's years of experience with radiochemistry coupled with Shearwater's expertise in synthesis and analysis of PEG derivatives gives Shearwater customers an excellent source of radio-labeled materials.

Please call our Sales team for pricing and availability.

Note On Purity

Shearwater's PEG reagents are purer than those obtainable from any other source, which is fitting since these reagents are intended for synthesis of pharmaceuticals and related biomedical products. The Company expends much effort purifying and characterizing PEG derivatives. One of the challenges of polymer derivatization is that polymeric by-products are difficult to remove because of the similar sizes and solubility of products and by-products. Shearwater's reagents are purified by precipitation/recrystallization and by chromatography where effective (see below).

For applications of monofunctional PEGs, small amounts of polymeric impurities are generally not of concern if the impurities are chemically inert and can be removed in subsequent steps. This statement does not hold when a difunctional derivative is used as a crosslinker since an inert end group leads to monosubstituted product that can be difficult to remove.

At Shearwater, reagent purity is determined by using one or more of the following methods: NMR, GPC, HPLC,TLC, FTIR, titration and ion-exchange chromatography. The results of these determinations are delivered with each reagent.

Most reagents are approximately 95% pure in terms of degree of end-group conversion. The prime impurity found in the Company's reagents is unmodified PEG. Small molecule contaminants are virtually nonexistent as determined by the above methods and by GC analysis.

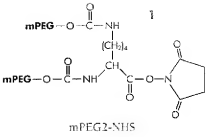
Specific comments regarding purity of individual reagents are included in the reagent descriptions

Storage Conditions For Active PEGs

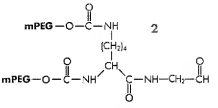
Derivatives such as the succinimidyl active esters (e.g., mPEG-SPA and mPEG2-NHS) are reactive with moisture. It is therefore essential to minimize exposure to moisture, and to keep the materials cold so that hydrolysis is retarded. Typical storage conditions are in an argon atmosphere at -20°C. Shipment at room temperature with no loss of activity is possible following thorough drying and storage under argon. Bottles containing reagent should be equilibrated at room temperature prior to opening to minimize condensation of moisture. Bottles can be opened in room air, but prior to closure and freezing, should be filled with dry inert gas (e.g. place the loosely capped bottle in a desiccator, pump down, and refill with argon). Repeated improper opening and closing will lead to significant loss of activity, and it is strongly recommended that samples be subdivided into small containers that are opened only once.

mPEG-ALD is readily oxidized by atmospheric oxygen to produce the corresponding carboxylic acid, and mPEG-THIOL reacts with oxygen to produce the disulfide (which can be reduced with DTT or borohydride). Storage under nitrogen is critical for these reagents.

All PEGs react slowly with oxygen to form peroxides along the backbone in a process that is facilitated by light and heavy metal ions. These peroxides can lead to chain cleavage. Therefore it is advisable to store all PEGs under nitrogen or argon, in the dark and as cold as possible, if the materials are to be stored for extended periods.



mPEG2-NHS



mPEG2-ALD

Shearwater's Activated PEG Reagents

PEG Architecture – Available Backbones

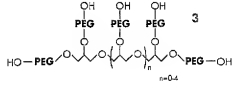
Polyethylene glycols are commonly available in two forms, difunctional PEGs and monofunctional methoxy-PEG (mPEG):

HO-(CH$_2$CH$_2$O)$_n$-CH$_2$CH$_2$-OH (PEG)

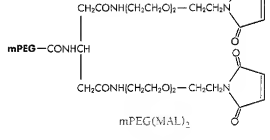
CH$_3$O-(CH$_2$CH$_2$O)$_n$-CH$_2$CH$_2$-OH (mPEG)

Activated derivatives of these linear PEGs are available in a variety of molecular weights, and details can be found in the following pages.

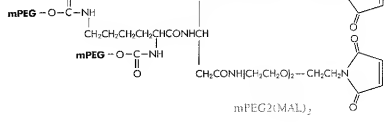
In addition to these linear PEGs, several different branched PEGs are available. These include monofunctional mPEG2 reagents (1 and 2), tetrafunctional 4-arm PEG (3), octafunctional 8-arm PEG (3), and difunctional forked mPEGs (4 and 5).



Multi-Arm PEG



mPEG(MAL)$_2$



mPEG2(MAL)$_2$

High Quality PEGs from NOF Corporation

$$HO\text{–}(CH_2CH_2O)_n CH_2CH_2\text{–}OH$$

PEG diol

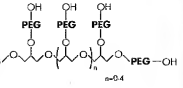
$$CH_3O\text{–}(CH_2CH_2O)_n CH_2CH_2\text{–}OH$$

low-diol mPEG

We maintain a supply of high quality, pharmaceutical grade PEGs manufactured by NOF Corporation of Japan. These PEGs are carefully prepared, purified and characterized and are well suited for preparing pharmaceuticals and biomedical products. The mPEGs contain only small amounts of diol, which is virtually undetectable in molecular weights below 10,000 Da and approximately 1% in molecular weight 20,000 Da. Higher molecular weights are also available. Polydispersities (MW/Mn) range from approximately 1.01 for mPEG 5,000 to 1.02 for mPEG 20,000. The PEG diol 20,000 from NOF is offered because it is difficult to obtain pharmaceutical grade PEG in these high molecular weights. MW/Mn is less than 1.20 for these high MW polymers. We do not stock low molecular weight PEG diols since these are available from a variety of sources.

Item No.	Description	Quantity	Price ($)
00000P02	PEG diol of MW 20K	1Kg	3,000.00
00000P52	PEG diol of MW 20K, Japanese Pharmacopeia grade	1Kg	1,200.00
2M000B01	Monomethyl ether of PEG (mPEG) MW1,000	1Kg	3,000.00
2M000D01	Monomethyl ether of PEG (mPEG) MW 2,000	1Kg	3,000.00
2M000E01	Monomethyl ether of PEG (mPEG) MW 3,000	1Kg	3,000.00
2M000H01	Monomethyl ether of PEG (mPEG) MW 5,000	1Kg	3,000.00
2M000L01	Monomethyl ether of PEG (mPEG) MW 10K	1Kg	6,000.00
2M000M01	Monomethyl ether of PEG (mPEG) MW 12K	1Kg	6,000.00
2M000P01	Monomethyl ether of PEG (mPEG) MW 20K	1Kg	6,000.00

Multi-Arm PEGs



Shearwater provides multi-arm, branched PEGs prepared by ethoxylation of various polyols (derived from glycerol condensation) having 4 to 8 arms.

Item No.	Description	Quantity	Price ($)
0J0000D04	4-arm PEG, MW 2,000	500g	1,500.00
		1Kg	3,000.00
0J0000D08	8-arm PEG, MW 2,000	500g	2,250.00
		1Kg	4,500.00
0J0000L03	3-arm PEG, MW 10,000	500g	1,500.00
		1Kg	3,000.00
0J000L04	4-arm PEG, MW 10,000	500g	1,500.00
		1Kg	3,000.00
0J000L08	8-arm PEG, MW 10,000	500g	1,500.00
		1Kg	3,000.00
0J0006N03	3-arm PEG, MW 15,000	500g	1,500.00
		1Kg	3,000.00
0J000CN04	4-arm PEG, MW 15,000	500g	1,500.00
		1Kg	3,000.00
0J000N08	8-arm PEG, MW 15,000	500g	1,500.00
		1Kg	3,000.00
0J0000P03	3-arm PEG, MW 20,000	500g	1,500.00
		1Kg	3,000.00
0J0000P04	4-arm PEG, MW 20,000	500g	1,500.00
		1Kg	3,000.00
0J0000P08	8-arm PEG, MW 20,000	500g	1,500.00
		1Kg	3,000.00
0J0000T08	8-arm PEG, MW 40,000	500g	2,250.00
		1Kg	4,500.00

Please call for availability.
Derivatives of Multi-Arm PEGs are also available.

GROUP ONE - NUCLEOPHILIC PEGs

mPEG-AMINE

$$mPEG\text{–}O\text{–}CH_2CH_2\text{–}NH_2$$

Polyethylene glycols with primary amino groups [1,2] at the termini are very useful functionalized polymers. The amino end groups on mPEG-AMINE are more reactive towards acylating agents than the hydroxyl groups that are present on conventional PEGs [12-15], and they also readily undergo reductive amination reactions [16, 17]. This reactivity offers a variety of methods for attachment of other molecules to the polymer via an array of stable linkages (e.g. amide, urethane, urea, secondary amine). Amine purity is approximately 90%. Chromatographically pure material is approximately 98% pure. These reagents can be used for:

a. preparation of conjugates with biologically active compounds [1-3, 14]
b. carrier for peptide synthesis [4-6]
c. preparation of PEG-coated surfaces and polymer grafts [7-9]
d. preparation of PEG-glycoprotein conjugates [10]
e. preparation of PEG-ligand conjugates for affinity partitioning [10]
f. preparation of PEG-cofactor adducts for bioreactors [10]

Item No.	Description	Quantity	Price ($)
2M2VDH01	mPEG-AMINE, MW 5,000	1g	21.00
		5g	84.00
2M2VDH21	mPEG-AMINE, MW 5,000 chromatographically pure	1g	42.00
		5g	168.00
2V2V0F02	AMINE-PEG-AMINE, MW 3,400	1g	21.00
		5g	84.00
2V2V0F22	AMINE-PEG-AMINE, MW 3,400 chromatographically pure	1g	42.00
		5g	168.00

GROUP TWO - ELECTROPHILICALLY ACTIVATED PEGs

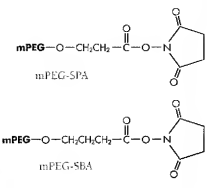
Derivatives of PEG bearing electrophilic groups reactive towards amines (such as lysines) and other nucleophiles are often referred to as activated PEGs. These PEGs are extensively used for attachment of PEG to proteins, liposomes, soluble and insoluble polymers, and a variety of molecules of biological relevance (commonly described as "PEGylation"). The monofunctional polymers are capped on one end by a methoxy group (mPEG) and are of particular importance because their reactions give products free of cross-linking (note that most of our mPEGs have virtually undetectable diol content). These reactions generally result in attachment of multiple strands of the polymer to the target molecule.

We offer a variety of quality, activated PEGs and mPEGs, including several NHS active esters, active carbonates and aldehyde. Our mPEG2 reagent is a high MW, monofunctional compound with the additional property of being sterically bulky, thus providing an effective "polymer cloud" around the modified molecule from a single point of attachment.

Choice of the appropriate reagent for a specific application is best made by considering a variety of properties such as: hydrolytic stability of the reagent; reactivity of the reagent; stability, toxicity and suitability for analysis. Details on these points are given in specific comments for each reagent. A brief comparison of coupling conditions and reactivity as well as a table of hydrolysis rates of NHS esters is given on page 12.

* cGMP Available

mPEG-Succinimidyl Propionate and mPEG-Succinimidyl Butanoate

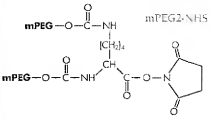


The NHS esters of PEG carboxylic acids are the most popular derivatives for coupling PEG to proteins. Reaction between lysine and terminal amines and the active esters produces a stable amide linkage. PEG succinimidyl succinate (mPEG-SS) is one of the oldest and most used PEG derivatives, but it possesses an ester linkage in its backbone and thus has the property of undergoing hydrolysis in vivo. mPEG-SPA and mPEG-SBA do not have an ester linkage in the backbone, they generate stable linkages therefore, and they have nearly ideal reactivity for protein modification (SPA is slightly more reactive than SBA). mPEG-SPA has been used in several human applications: mPEG-SPA 5,000 has been used for attachment to a protein antagonist, which has successfully completed clinical trials and an NDA has been filed (the mPEG-SPA 5,000 process is validated and suitable for commercial use). mPEG-SBA 30,000 is currently being used in Phase I clinical trials as an adduct to a powerful protein as a second generation product. Results from this Phase I study look very promising. mPEG-SPA and mPEG-SBA are approximately 95% pure.

See references #20, 21

Item No.	Description	Quantity	Price ($)
2M4M0D01	mPEG-SPA, MW 2,000	500 mg	100.00
		1 g	200.00
		5 g	800.00
2M4M0H01 *	mPEG-SPA, MW 5,000	500 mg	90.00
		1 g	180.00
		5 g	720.00
2M4M0P01	mPEG-SPA, MW 20,000	500 mg	110.00
		1 g	220.00
		5 g	880.00
4M4M0F02	SPA-PEG-SPA, MW 3,400	500 mg	90.00
		1 g	180.00
		5 g	720.00
2M450H01	mPEG-SBA, MW 5,000	1 g	190.00
		5 g	760.00
4545DF02	SBA-PEG-SBA, MW 3,400	1 g	230.00
		5 g	920.00

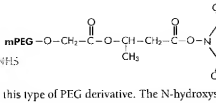
mPEG2-N-Hydroxysuccinimide



We have attached mPEG to lysine to obtain a branched acid, mPEG2-COOH. Activation with N-hydroxysuccinimide gives the active ester mPEG2-NHS[P274], mPEG2-NHS has several advantages. First, the compound is purely monofunctional because the intermediate acid is purified by ion exchange chromatography. Second, the branched structure of the compound results in a relatively large molecular volume, so that the advantages of PEG attachment can be obtained without as many points of attachment. Also, our experience has shown that the mPEG2 reagent is less likely to penetrate into sterically crowded regions of a protein. Although the hydrolysis rate and rate of reaction with small amines is faster than most NHS esters (Table, Pg. 12) the rates of reaction with proteins are slower, presumably because of the large steric effects involved; slightly increased reaction times are necessary. This mPEG2 is also available as the aldehyde (Pg. 7) and the maleimide (Pg. 8). The mPEG2-NHS 40,000 reagent is being used in several clinical trials for attachment to several proteins, such as Hoffmann-La Roche's PEGASYS (PEGylated-interferon alfa). The PEGASYS product completed clinical trials and has been filed with the FDA. The mPEG2-NHS 40,000 reagent is a validated commercial quality PEG derivative.

Item No.	Description	Quantity	Price ($)
2Z3X0L01	mPEG2-NHS ester MW 10,000	500 mg	150.00
		1 g	320.00
		5 g	1280.00
2Z3X0P01	mPEG2-NHS ester MW 20,000	500 mg	160.00
		1 g	320.00
		5 g	1280.00
2Z3X0T01 *	mPEG2-NHS ester MW 40,000	500 mg	160.00
		1 g	320.00
		5 g	1280.00

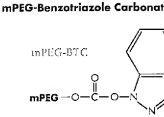
mPEG-Double Esters



There are two ester linkages in this type of PEG derivative. The N-hydroxysuccinimidyl (or NHS) ester of mPEG-CM-HBA-NHS is the most active ester, and readily reacts with amino groups on proteins or other amine-containing molecules under mild conditions. The second ester (between CM and HBA) provides the conjugate degradability by hydrolysis in aqueous media. The derivative has been used in coupling to an enzyme[225] and in forming PEG hydrogels[170].

Item No.	Description	Quantity	Price ($)
OW0WOF02	NHS-HBA-CM-PEG-CM-HBA-NHS, MW 3,400	500 mg	175.00
		1 g	350.00
OW2MOH01	mPEG-CM-HBA-NHS, MW 5,000	500 mg	175.00
		1 g	350.00

mPEG-Benzotriazole Carbonate



The benzotriazole carbonate derivative of mPEG (mPEG-BTC) has been added to the Shearwater reagent list as an exceptional alternative to the succinimidyl carbonate (mPEG-SC). While not as reactive as some of the NHS active esters (pg. 12), mPEG-BTC is an efficient modifier of peptide and protein amino groups, producing a stable urethane (carbamate) linkage. mPEG-BTC is sufficiently reactive to produce extensively modified PEG-proteins under mild conditions within short periods of time. The mPEG-BTC is an intermediate for several GMP syntheses and is expected to begin GMP synthesis as a final product in 2001. 5,000 and 20,000 Da have been the most commonly used molecular weights. Purity is >95%. See reference #27

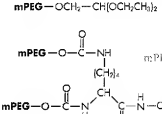
Item No.	Description	Quantity	Price ($)
OM2MOH01	mPEG-BTC, MW 5,000	1 g	105.00
		5 g	420.00
OM2MOP01	mPEG-BTC, MW 20,000	1 g	120.00
		5 g	480.00
OM0M0F02	BTC-PEG-BTC, MW 3,400	1 g	105.00
		5 g	420.00

mPEG-Propionaldehyde

PEGs bearing aldehyde groups undergo reductive amination reactions with primary amines in the presence of sodium cyanoborohydride. Unlike other electrophilically activated groups, the aldehyde reacts only with amines. Although aldehyde is much less reactive than the NHS esters, this reaction takes place under mild conditions (pH 6-9.5, 6-24 hours) and has been shown to be useful for attaching PEG to surfaces[28] and proteins[29, 30]. At lower pH, selective reaction at the N-terminus becomes possible. The stability of the attachment (a secondary amine is formed upon reduction) is important for such applications as preparation of affinity supports and immobilized enzymes. Proteins modified in this fashion retain amine groups and associated charge in solution, which can be important for maintaining protein conformation and activity. These conjugates can be conveniently characterized by quantitation of lysine in their hydrolysis products by amino acid analysis. mPEG-ALD has also been used to form acetal linkages with hydroxyl groups of polyvinyl alcohol[171]. The propionaldehyde reagent offered here has the advantage of being much more stable in basic media than the acetaldehyde reagent[31-33]. mPEG-ALD is very popular for N-terminal PEGylation of proteins and two of these, 20 and 30 kDa, are being used in Phase III and Phase II clinical trials with two different proteins. Like the manufacture of other Shearwater reagents, these can be produced cost effectively. Purity is approximately 80%. See references 328 - 34

Item No.	Description	Quantity	Price ($)
OS2MOH01	mPEG ALD, MW 5,000	1 g	160.00
		5 g	640.00
OS2MOP01 [A]	mPEG-ALD, MW 20,000	1g	160.00
		5g	640.00
OS2MOR01 [A]	mPEG-ALD, MW 30,000	1g	200.00
		5g	800.00
OS0S0F02	ALD-PEG-ALD, MW 3,400	1g	160.00
		5g	640.00

mPEG-Acetaldehyde Diethyl Acetal and mPEG2-Aldehyde

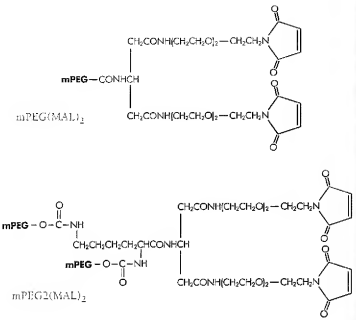


mPEG-ALD has the advantage over mPEG-acetaldehyde of being stable toward aldol condensation. Nonetheless, mPEG-ACET can be stored as the diethyl acetal and hydrolyzed in situ to produce the active aldehyde and hydrate[32a,32b]. High yields of conjugate with proteins can be obtained via reductive amination. Similarly mPEG2-ALD is also a useful compound[227]. In this case, loss of aldehyde via aldol condensation is retarded by steric hindrance. High quality mPEG2-ALD is available in molecular weights up to 60,000 as a custom item.

Item No.	Description	Quantity	Price ($)
042MOH02	mPEG-ACET, MW 5,000	1 g	320.00
		5 g	1280.00
053X0T01	mPEG2-ALD, MW 40,000	500 mg	400.00
		1 g	800.00
		5 g	3200.00

GROUP THREE - SULFHYDRYL-SELECTIVE PEGs

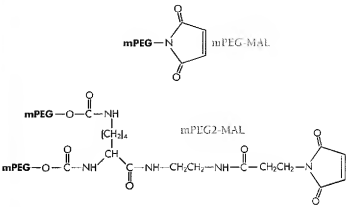
mPEG-Forked Maleimide and mPEG2-Forked Maleimide



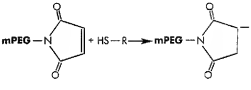
Most PEGylation has been performed with linear PEGs. However, PEGs can be constructed with many different architectures. Additional structures, which are now available, are the "forked" PEGs shown above[139]. These structures have the advantage of placing two reactive groups at a precise distance apart. The above PEGs have become very popular for mimicking the heavy chain domain in an antibody and other applications where two proteins (alike or different) in proximity are advantageous. Shearwater has one partner with a forked mPEG2 in Phase II clinical trials. We have synthesized many variants of mPEG2, with a range of molecular weights and reactive groups.

Item No.	Description	Quantity	Price ($)
2D2D0H0F	mPEG(MAL)2, MW 5,000	500 mg	175.00
		1 g	350.00
2D2D0P0F	mPEG(MAL)2, MW 20,000	500 mg	175.00
		1 g	350.00
2D3X0T0F	mPEG2(MAL)2, MW 40,000	500 mg	200.00
		1 g	400.00

mPEG-Maleimide and mPEG2-Maleimide



Coupling of maleimide and thiol groups is one of the most useful reactions for bioconjugate preparation. This reaction is highly specific and takes place under mild conditions in the presence of other functional groups. mPEG-MAL has been used as a reactive polymer for preparing well defined, bioactive PEG-protein conjugates[137,47]. mPEG-MAL has also been used as a polymeric reagent for selective entrapment of thiol-containing peptides[60]. Unlike PEG vinylsulfone, maleimide reacts slowly with water by addition across the double bond. Also, slow cleavage of one of the amide linkages of the conjugate may occur[142]. Two versions of our sulfhydryl-selective chemistry, mPEG-MAL 5,000 and mPEG2-MAL 40,000, are in Phase II clinical trials. Purity is approximately 80%.

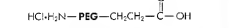


Item No.	Description	Quantity	Price ($)
2D2M0H01*	mPEG-MAL, MW 5,000	1 g	150.00
		5 g	500.00
2D2M0P01	mPEG-MAL, MW 20,000	1 g	150.00
		5 g	600.00
2D2D0F02	MAL-PEG-MAL, MW 3,400	1 g	150.00
		5 g	600.00
2D3X0T01*	mPEG2-MAL, MW 40,000	500 mg	235.00
		1 g	470.00
		5 g	1880.00

* cGMP Available

GROUP FOUR-HETEROFUNCTIONAL PEGs

Amines and Acids



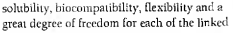
NH2-PEG-COOH

Heterofunctional derivatives of PEG have the general structure X-PEG-Y and are extremely useful as macromolecular crosslinking agents or as spacers between two different entities[42-46]. In addition to the two independent functional groups, the presence of PEG provides water solubility, biocompatibility, flexibility and a great degree of freedom for each of the linked components of X and Y. Examples reported in the literature include: synthesis of enzymatically active cofactor-apoenzyme conjugates[47], preparation of graft polymeric supports for solid-phase peptide synthesis[48], targetable polymeric drugs[49] and PEG-grafts on surfaces and proteins[50].

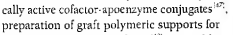
We offer heterofunctional PEGs with an NHS ester on one end and vinylsulfone or maleimide on the other, t-Boc-protected amines, the active PEGs with biotin at one terminus (following pages) and ω-amino-α-carboxyl PEG above (approximately 95% purity). These end groups can be converted to other functional groups, depending upon the user's requirements.

Item No.	Description	Quantity	Price ($)
022V0F02	HCl-NH2-PEG-COOH		
	MW 3,400	500 mg	275.00
		1 g	550.00
		5 g	2200.00

FMOC and t-Boc-Protected Amines



t-Boc-PEG-NHS

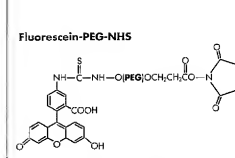


FMOC-PEG-NHS

The above heterofunctional PEGs offer many possibilities for tethering, crosslinking and conjugation. Typically the NHS ester is first coupled in water. Deprotection and coupling of the amine is then performed. The t-Boc protecting group can be easily removed by treatment with trifluoroacetic acid (TFA). FMOC-PEG-NHS is provided for our customers who prefer FMOC protection. Our materials are approximately 90% pure as shown by TLC, GPC, NMR and ion exchange chromatography. Molecular weight is 3,400 for both derivatives. Other molecular weights can be prepared as custom items.

Item No.	Description	Quantity	Price ($)
22b30F02	t-Boc NH PEG-CO2NHS		
	MW 3,400	500 mg	210.00
		1 g	420.00
		5 g	1680.00
1P220F02	FMOC-NH-PEG-CO2NHS		
	MW 3,400	500 mg	210.00
		1 g	420.00
		5 g	1680.00

Fluorescein-PEG-NHS

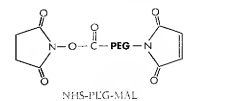


Fluorescein-PEG-NHS

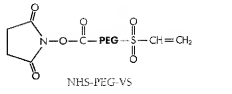
Fluorescein-PEG-NHS has a fluorescein moiety at one end of a PEG chain and an NHS ester at the other. This heterofunctional derivative can be coupled to proteins and other amine-containing molecules, and provides an excellent means of monitoring attachment. Thus, this reagent can serve as a compliment to fluorescamine or TNBS. An advantage of fluorescein-PEG-NHS is that it has the same reactivity as the commonly used mPEG-SPA.

Item No.	Description	Quantity	Price ($)
1K220D02	Fluorescein-PEG-NHS		
	MW 2,000	500 mg	210.00
		1 g	420.00
		5 g	1680.00
1K220H02	Fluorescein-PEG-NHS		
	MW 5,000	500 mg	210.00
		1 g	420.00
		5 g	1680.00

NHS-Vinylsulfone and NHS-Maleimide

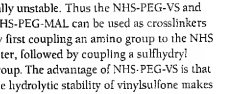


NHS-PEG-MAL



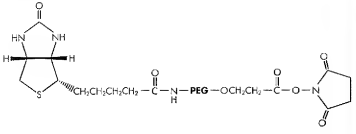
NHS-PEG-VS

NHS-PEG-MAL and NHS-PEG-VS are especially useful heterofunctional PEGs. The vinylsulfone and maleimide groups are selective for reaction with sulfhydryl groups around pH 7. Reaction with amino groups proceeds at higher pH, but is still relatively slow. Also, the vinylsulfone group is hydrolytically stable, while maleimide shows some reactivity with water. Maleimide is more reactive than vinylsulfone and sometimes works when vinylsulfone will not. The NHS ester group is highly reactive toward amino groups, but is hydrolytically unstable. Thus the NHS-PEG-VS and NHS-PEG-MAL can be used as crosslinkers by first coupling an amino group to the NHS ester, followed by coupling a sulfhydryl group. The advantage of NHS-PEG-VS is that the hydrolytic stability of vinylsulfone makes possible a leisurely approach to the second step. Approximate purity of each end group is >80%.

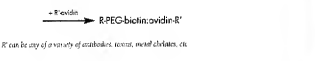


Item No.	Description	Quantity	Price ($)
27560F02	NHS-PEG-VS		
	MW 3,400	100 mg	130.00
		500 mg	650.00
		1 g	1040.00
2D220F02	NHS-PEG-MAL		
	MW 3,400	100 mg	105.00
		500 mg	525.00
		1 g	840.00

GROUP FIVE – PEG-BIOTIN



Conjugation of moieties utilizing the high binding strength and specificity of biotin-avidin association is well known. PEG offers advantages as a tether in this application since PEG is nontoxic and highly water soluble. Attachment of PEG can result in aqueous solubility for molecules that are normally not water soluble. PEG can also provide reduction in immunogenicity. Since avidin is multivalent, several PEGs can be tethered in this way. The heterofunctional biotin PEGs provide a ready approach for attaching PEG-biotin to an aminated molecule or surface (for biotin-PEG-NHS). The resulting conjugates can then be coupled to avidin-containing molecules or surfaces. Approximate purity of each end group is >80%.



Where R and R' can be any of a variety of attachables, terminal chelates, etc.

Item No.	Description	Quantity	Price ($)
01220F02	Biotin-PEG-CO2-NHS MW 3,400	100 mg	100.00
		500 mg	500.00
		1 g	800.00

GROUP SIX - VINYL DERIVATIVES

$$CH_2{=}CHCO_2{-}\mathbf{PEG}{-}CO_2{-}NHS$$

ACRL-PEG-NHS

Acrylates offer the possibility of vinyl polymerization or co-polymerization to produce graft polymers or cross-linked materials with excellent properties for biomaterial applications. Such materials are resistant to protein and cell adhesion and upon breakdown yield nontoxic degradation products[50,51]. The ACRL-PEG-NHS ester offers a route for inclusion of enzymes in acrylic polymers[52]. Purity is approximately >85%. The reagent contains less that 200 ppm of 4-methoxyphenol as an inhibitor. The acrylate material is light sensitive and will cross-link with exposure to UV light.

Item No.	Description	Quantity	Price ($)
01220F02	ACRL-PEG-NHS, MW 3,400	250 mg	120.00
		500 mg	240.00
		1 g	384.00

GROUP SEVEN – PEG-PHOSPHOLIPIDS

$$RCO_2-CH_2-CH[O_2CR]-CH_2-OPO_3CH_2CH_2NH-CO_2-\mathbf{mPEG}$$

mPEG-DSPE

There has been intense interest in use of liposomes for controlled-release and selective-delivery of drugs. A problem with this application is that liposomes, especially larger ones, are quickly cleared from the body. Recent research has shown that incorporation of PEG into the outer coating of liposomes can greatly increase plasma lifetime, thus solving a critical problem blocking application of this promising drug delivery technique[53]. PEGylated liposomes are made by two general methods, one in which PEG-phospholipid is used to form a liposome [54,55], and a second in which liposomes with attached amino groups are reacted with suitably activated PEG [56]. We maintain a supply of distearoylphosphatidylethanolamine modified with mPEG 5000 or 2000 (abbreviated DSPE-5000 and DSPE-2000). Purity is approximately 98% + by NMR and HPLC.

Shearwater has developed methods for synthesizing highly pure homo and heterobifunctional PEG phospholipids. Shearwater now provides these reagents in research and cGMP quantities. Among the different phospholipid types are the lauryl, myristoyl, palmitoyl, stearoyl, oleoyl and linoleoyl analogs of 1,2-diacyl-sn-glycero-3-phosphatidyl ethanolamine. We can supply these as mPEG-phospholipids or as activated heterobifunctional X-PEG-phospholipids where X = maleimide or N-hydroxysuccinimidyl carbonate. If you have a specific request, please contact our Sales team. *See references #53 – 59*

Item No.	Description	Quantity	Price ($)
172M0D01	mPEG-DSPE, MW 2,000	500 mg	115.00
		1 g	230.00
		5 g	920.00
172M0401	mPEG-DSPE, MW 5,000	500 mg	115.00
		1 g	230.00
		5 g	920.00
172D0F02	DSPE-PEG-MAL, MW 3,400	500 mg	440.00
		1 g	880.00
		5 g	3,520.00
172201F02	DSPE-PEG-NHS, MW 3,400	500 mg	440.00
		1 g	880.00
		5 g	3,520.00

BRIEF REVIEW OF PEG APPLICATIONS

At least eight emerging technologies require active PEGs. A full technical description of these technologies is beyond the scope of this catalog, and the reader is referred to the following volumes for more extensive discussion and leading references: "Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications", J. M. Harris, Ed., Plenum, NY, 1992 and "Poly(Ethylene Glycol) Chemistry and Biological Applications", J. M. Harris and S. Zalipsky, Ed., ACS, Washington, 1997. Note: these books are available from Amazon.com or Shearwater Corporation–please call our Sales team for pricing.

A key property of PEG is that attachment to other molecules and surfaces provides a biocompatible, protective coating. This protective coating slows rejection of materials in biological systems (such as the human body), greatly reduces protein, cell and bacterial adsorption, and reduces the rate of kidney clearance (because of larger size). PEG also is nontoxic and has been approved by the FDA for topical and internal use in humans. PEG is soluble in water and many organic solvents, and it forms aqueous two-phase systems when paired with certain other polymers (such as dextran). It is insoluble in ethyl ether and hydrocarbons such as hexane. The water solubility, lack of toxicity, high flexibility and well-defined chemistry of bifunctional PEG makes it ideally suited for many crosslinking or tethering applications.

Eight technologies that result from use of these properties are: PEG-proteins, peptides and small molecules for pharmaceutical use; PEG-surfaces for electrically-controlled, nonfouling materials; PEG-liposomes for drug delivery; molecule-molecule or molecule-surface coupling for drug and materials applications; PEG-molecules for biological purifications; biopolymer synthesis on PEG supports; PEG attachment for control of solubility (e.g., enzymes into organic solvents or water solubilization of enzyme substrates, dyes, flavors and chemotherapeutic agents); and hydrogels for drug delivery and wound coverings.

PEG-Proteins for Pharmaceutical Use

It has been demonstrated that proteins with PEG attached (i.e., PEG-proteins, made by reaction of the protein with an active PEG) remain active and have a greatly diminished or negligible immune response. The result is that these PEG-proteins have greatly increased plasma lifetimes. Examples include mPEG-IFN-A, mPEG-IFN-B, mPEG-IL-2 and mPEG-GCSF. In addition, PEG attachment makes proteins much larger and thus reduces their rate of clearance through the kidney. PEG has also been attached to many small molecules (such as vitamin E, cholesterol, fluorouracil, etc.). The goal here is to reduce the rate of kidney clearance and impart water solubility.

PEG-Surfaces

In addition to the molecular modifications, PEG can also be attached to surfaces to form protective, biocompatible coatings. A variety of applications result, including PEG-coatings for arterial replacements, diagnostic apparatus and blood contacting devices. Similarly, capillary zone electrophoresis has emerged as an important new analytical technique in biochemistry, and PEG coatings on the capillaries prevent protein adsorption and provide critical control of electroosmosis.

PEG-Liposomes

There has been intense interest in use of liposomes for controlled-release and selective-delivery of drugs. A problem with this application is that liposomes, especially larger ones, are quickly attacked and cleared from the body. Recent research has shown that incorporation of PEG into the outer coating of liposomes can greatly increase serum lifetime, thus solving a critical problem blocking application of this promising drug delivery technique.

Molecule-Molecule and Molecule-Surface Coupling

The hydrophilic, biocompatible nature of PEGs and their mild, well-defined chemistry makes them ideal for coupling or tethering molecules to molecules or molecules to surfaces. This technology is critical for the next generation of drugs and biomaterials. Research has shown that use of PEG as a coupler to bind molecules to other molecules and surfaces provides highly active materials.

Biological Purification

The genetic engineering revolution has led to methods for production of a variety of physiologically active proteins. There is, however, a critical need in this industry for improved methods for isolation of the proteins produced. An approach to this problem that has recently received much interest is purification by partitioning in aqueous two-phase systems (analogous to oil and water) made by solution of PEG, other polymers and salts in water. In this approach, a PEG-ligand is made (such as a PEG-antibody), which binds specifically with the desired protein and pulls the protein into the PEG-rich phase.

Biopolymer Synthesis

The three bio-oligomers (peptides, oligonucleotides, and oligosaccharides) can all be grown on PEG as a soluble carrier. The PEG-oligomer is precipitated after each step to isolate the product, which can then be cleaved or taken to the next addition step. Advantages of this method are that fewer errors result, chemistry is faster, and large quantities of materials can be handled. A variation on this theme is to build the bio-oligomer on a PEG chain that is bound to a solid polystyrene particle. This approach apparently provides advantages of both solid-phase and liquid-phase synthesis.

Solubilization of Insoluble Molecules

PEG is soluble both in water and in many organic solvents. This property has been utilized to solubilize other molecules by PEG attachment. An interesting biotechnical application is solubilization of enzymes in organic solvents such as chlorinated hydrocarbons. Additionally, water-insoluble materials may become water-soluble after PEG attachment. Examples here include dyes, flavors, substrates for enzymes, cofactors, pharmaceuticals, etc. Shearwater has developed proprietary technology for controlled release of PEG-bound drugs. Please call to discuss this application.

PEG-based Hydrogels

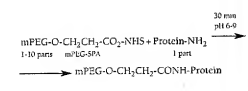
Multifunctional PEGs can be cross-linked in a variety of ways to make hydrogels. These hydrogels have many uses and are especially of interest for controlled release of drugs and for wound coverings. Shearwater has proprietary technology for preparation of PEG hydrogels. Please call to discuss this application.

Conditions for Protein PEGylation

Reaction conditions for coupling PEG to a protein vary depending on the protein, the desired degree of PEGylation and the PEG derivative utilized. Factors involved in choice of derivative are: desired point of attachment (lysine vs. cysteine); hydrolytic stability and reactivity of the derivative; stability of the linkage; and suitability for analysis. Details on these points are given in specific comments for each derivative.

Lysine-Active PEGs

The most frequently used derivatives for lysine attachment are the N-hydroxylsuccinimide (NHS) active esters such as PEG succinimidyl succinate (mPEG-SS) and succinimidyl propionate (mPEG-SPA). Typically several PEGs can be attached to a protein at pH 8-9.5, room temperature, within 30 minutes, if equal masses of PEG (MW 5,000) and protein are mixed, although for some proteins it is necessary to add a 10-fold mass of PEG relative to the protein. If the protein amino acid composition is known, a molar ratio of PEG (MW 5,000) to protein amino groups of 1-5 to 1 will usually suffice. Increasing pH increases the rate of reaction and lowering pH reduces rate of reaction. These highly reactive "active esters" can couple at physiological pH, but the less reactive derivatives typically require high pH. Low temperatures may also be used if a labile protein is under study. In this case, a long reaction time may be useful. Analysis of several reactions with varying ratios of PEG/protein and with varying pH will quickly provide information sufficient to design conditions giving desired degrees of PEGylation.



Using mPEG-SS and mPEG-SPA as a reactivity baseline, one can make educated guesses about reaction conditions for the other lysine-active derivatives. See the tables at the end of this section for relative reactivity of lysine-active derivatives. For general discussion of this subject see S. Zalipsky and C. Lee[ref].

Another factor that must be considered in choosing reaction conditions is rate of hydrolysis relative to aminolysis. All of the lysine-active derivatives react with water, except for aldehyde; epoxide hydrolysis is relatively slow at pH 9, but does occur. Primary amine is much more nucleophilic than water, so aminolysis is always faster than hydrolysis. From the limited studies that have been done, it appears that aminolysis selectivity increases with an increase in pH.

All of the lysine-active derivatives, except aldehyde, can possibly react with other amino acids, such as imidazole groups of histidine and hydroxyl groups of tyrosine, if the local environment enhances nucleophilicity of these groups. Aldehyde is selective for amines.

Table 1

Comparison of reactivity of various PEG derivatives toward protein lysine groups

	Lysine Reactivity Relative to mPEG-SS or mPEG-SPA (pH 8-9)			
PEG Derivative	*Similar*	*Faster*	*Slower*[b]	*Much Slower*[c]
carboxymethyl-NHS		X[a]		
norleucine-NHS		X[d]		
succinimidyl carbonate	X			
tresylate				X
aldehyde				X
epoxide				X
carbonylimidazole				X
PNP carbonate			X	

[a]Reaction over in 1-2 minutes [b]Try 1-2 hours [c]Try 18-24 hours [d]Try 5-10 minutes

Table 2

Hydrolysis half lives at pH 8, 25 °C, for PEG active esters measured by following UV absorbance of succinimidyl (NHS) group or benzotriazole (BTC) group.

PEG-NHS Ester	*symbol*	*half life (minutes)*[b]
mPEG-O-CH_2CH_2-CO_2-NHS	SBA	23.3
mPEG-O-CO_2-NHS	SC	20.4
mPEG-O_2C-$CH_2CH_2CH_2CO_2$-NHS	SG	17.6
mPEG-O-CH_2CH_2-CO_2-NHS	SPA	16.5
mPEG-O-CO_2-BTC	BTC	13.5
mPEG-S-CH_2CH_2-CO_2-NHS	SSPA	10.7
mPEG-O_2C-CH_2CH_2-CO_2-NHS	SS	9.8
mPEG-O_2CNH-CH(Bu)-CO_2-NHS	NOR	5.4
mPEG-O_2CNH-CH(R)-CO_2-NHS[a]	mPEG2	4.9
mPEG-NHCO-CH_2CH_2-CO_2-NHS	SSA	3.2
mPEG-O-CH_2-CO_2-NHS	SCM	0.75

[a] R = -$CH_2CH_2CH_2CH_2$-CO_2-NHCO$_2$-PEG [b] As a rough rule of thumb the half life triples upon lowering pH one unit.
* Aminolysis rates parallel hydrolysis rates

Sulfhydryl-Selective PEGs

Shearwater offers two sulfhydryl-selective PEGs: vinylsulfone and maleimide. Typical reaction conditions for these derivatives are pH 7-8, slight molar excess of PEG, and 0.5-2 hour reaction at room temperature. For sterically hindered sulfhydryl groups, required reaction times may be significantly longer.

Analysis of PEG-Proteins

There are at least three aspects to be considered here: first, how many PEGs have been attached to a protein; second, which amino acids have been PEGylated and to what extent; and third, how many different PEG-protein forms, and how much of each, are present in a sample. The first question can be answered with some precision using readily available equipment, but the second and third questions remain very much a matter for research and require use of highly sophisticated equipment such as tandem mass spectrometers.

It should be noted that a PEGylated protein is typically a mixture of varying numbers of PEGs of varying molecular weights attached to several amino acid sites. Each starting PEG sample used to prepare a particular reagent contains a range of PEGs of varying molecular weight (typical polydispersity, MW/Mn, values are < 1.05), so proteins containing the same number of PEGs will have a range of molecular weights. And these PEGs will be distributed on different amino acids. In addition, a statistical distribution of numbers of attached PEGs is obtained.

For the usual situation in which the PEGylation takes place on lysine, a common analytical approach is to determine the number of lysine groups that have been modified. The usual method for performing this analysis is reaction of unmodified lysine amino groups with trinitrobenzenesulfonic acid followed by UV measurement (the "Habeeb method")[61,62]. This method requires several milligrams of protein, and it is harmed by the presence of unattached PEG. Protein concentration must also be measured (Biuret works well). A superior approach is the fluorescamine method of Stocks[64, 65]. This method works by reaction of fluorescamine with unmodified lysine groups to give a fluorescent derivative. Advantages are that free PEG does not interfere, and only nanogram amounts of protein are required. A recent refinement of this method has been described[65]. The Habeeb and fluorescamine methods have proven to be very useful for monitoring PEGylation with lysine-active PEGs.

A related spectrophotometric method for determining available cysteine sulfhydryl groups is based on reaction with 2,2'-dipyridyl disulfide to form 2-thiopyridone, which absorbs at 343 nm with ε = 7060 at pH 7.2[65]. Another effective approach is reaction with Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid)][67].

A more exact method for monitoring reacted lysines is to couple mPEG fluorescein to the protein. Hydrolysis followed by amino acid analysis provides a direct measure of the fluorescein content and thus a measure of the number of PEGs attached[66,68]. Fluorescein can also be measured directly on the PEGylated protein. Amino acid analysis will also work for those PEG derivatives that modify amino acids through hydrolytically stable linkages, an example of which includes mPEG-ALD which couples to lysines through secondary amine linkages. In this case, the number of bound PEG chains is calculated on the basis of lysine decrease.

Amino acid analysis can also be used for coupling of mPEG-VS to cysteine groups since a hydrolytically stable thioether linkage is formed. In this case, the degree of substitution can be calculated from reduction in cysteine groups as measured by amino acid analysis.

The amount of PEG can also be determined by proton NMR[69] and by use of ^{14}C labeled PEG[69-1]. Measurement of PEG-protein molecular weight would also provide this same information, but unfortunately most of these methods (e.g., gel filtration chromatography and gel electrophoresis) do not provide quantitative information because PEG "acts" much larger than the proteins used for calibration[72, 73]. However, these sizing techniques are extremely useful for qualitative analysis of PEG proteins because they demonstrate the occurrence of PEGylation and they are very effective in monitoring reproducibility of preparations. Dollinger et al. have used HPLC with a fluorimeter detector performing as a light scattering detector to provide on-line molecular weight determination of PEG-proteins[74].

Determination of overall heterogeneity in a PEG-protein (i.e., number of PEG-protein forms present) remains difficult and is certainly not a routine matter[72,73]. A variety of chromatography and electrophoresis methods have been applied to this problem, and these papers provide an excellent review of this subject. The question of sites of PEG attachment and amount of PEGylation at each site has been addressed using mass spectrometery[75, 76] and NMR spectroscopy[77]. These studies show that certain lysines are more available for attack than others, and that histidines and tyrosines are subject to attack by unselective reagents such as the dichlorotriazines. It is also clear that generalizations are risky and one should watch for protein-dependent exceptions.

One of the most significant recent advances in PEG and PEG-protein analysis has been the availability of matrix-assisted-laser-desorption-ionization mass spectrometry (MALDI-MS)[70,80]. MALDI provides the mass of the unfragmented, singly-charged molecular ion of macromolecules up to about 100,000 Da, and thus greatly assists determination of polydispersity and identity of PEG derivatives. Similarly, the mass of the molecular ions of PEG conjugates, such as PEG-proteins, can also be determined, and the composition of PEGylation reaction products containing different numbers of PEGs ("one-mer", "two-mer", etc.) can be established.

Capillary electrophoresis (CE) is another powerful analytical method that is increasingly being used for analysis of PEG-proteins. Recent work has shown that this method can be used to determine the amount of each PEG-mer in a PEG-protein[71]. CE can also be used in analysis of fragmented PEG-proteins for determination of the site of PEGylation.

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